Patent
Attorney's Docket No. 1017753-000235

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	**Mail Stop AMENDMENT**
Bertrand PAIN et al.	)	Group Art Unit: 1633
Application No.: 10/625,847	)	Examiner: S. Kaushal
Filed: July 24, 2003	)	Confirmation No.: 8939
For: AVIAN CELL LINES USEFUL FOR THE PRODUCTION OF SUBSTANCES OF INTEREST	)	

DECLARATION UNDER 35 U.S.C. § 1.132 OF DR. FABIENNE GUEHENNEUX

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Fabienne Guéhenneux, declare the following:

2. I am a citizen of France, and have the following mailing address: 4 rue de la close du Ruaud, Le Temple de Bretagne, France;

3. I graduated from University Claude Bernard with a Ph.D. degree in Cell Biology and Immunology;

4. I am the Head of Cell Biology Department of Vivalis, and have held this position since the year 1999;

5. I am skilled in the art of culturing embryonic stem cells, more specifically avian embryonic stem cells;

6. I am the inventor of the above-identified United States Patent Application, and I am submitting this Declaration in support of that application;

7. Chicken cell lines have been obtained by a process described in the present Declaration;

8. I have performed and/or supervised the experiments reported below:

1. Establishment of chicken EB1 cell line

1.1 - RAW MATERIAL

Eggs

Eggs used for this project of development were produced by the S86N chicken strain produced by the Grimaud group. The S86N strain is the result of a cross between P6N females and S88N males. Those eggs were not Specific Pathogen Free (SPF) eggs but, thanks a constant follow up of chicken flocks and strict procedures of access to production site, risk of cross contamination were under control. The chickens used for the egg production were vaccinated against Avian Infectious Bronchitis, Infectious Bursal disease, Marek Disease, Newcastle Disease, Rhinotracheite infectieuse, Laryngothracheitis, Encephalomyelitis, EDS76 (Adenovirus), Avian Coronavirus, Gumboro and Chicken Anemia (CAV). After vaccinations, sanitary controls were performed to check immunity against chicken anemia and Encephalomyelitis. During egg production, regularly, others controls have taken place to check absence of salmonella, Mycoplasma synoviae and Mycoplasma gallisepticum.

Feeder cells

In the first step of the process of establishment of EB1, genetically engineered cells from murine origin (STO cells) were used as feeder layer to maintain the pluripotency of chicken stem cells. Those feeder cells are mitotically inactivated by gamma irradiation (45 to 55 Grays) before seeding on plastic. This dose of irradiation is a sub-lethal dose that induces a definitive arrest of the cell cycle but still permits the production of growth factors and extra-cellular matrix, necessary for the promotion of the cell growth of non differentiated cells.

The STO cell line was derived by A. Bernstein, Ontario Cancer Institute, Toronto, Canada from a continuous line of SIM (Sandos Inbred Mice) mouse embryonic fibroblasts and it was supplied by the American Type Culture Collection (ATCC) (STO Product number: CRL-1503, Batch number 1198713). Those STO cells were modified by transfection for the production of cell lines able to secrete in supernatant GPAR (Growth

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Promoting activity receptor) (SGN cells) or SCF (SCN cells). Fresh feeder layers were prepared twice a week, in general on monday and thursday. Exponentially cells were dissociated and counted. A part of cells were seeded for maintenance of viable cultures and another part was irradiated. For irradiation, we prepared a cell suspension at $10x10^6$ cells/mL in tubes. Cells were exposed to a 45 to 55 grey dose and were seeded on plastic. After seeding, dishes or plates coated with inactivated feeder cells (50% of SGN cells and 50% of SCN cells) were used during a maximum of 5 days.

Medium

MacCoy 5'A (Invitrogen, Cat.26600-023)
Optipro medium (Invitrogen, Cat n° 12309)

Additives

Glutamine (Biomedia, Cat n° GLUN2002012)
Pencillin/streptomycin (Biomedia, Cat n°PEST1002012))
Non essential Amino Acids (Biomedia Cat n° AANE2012)
Sodium pyruvate (Biomedia, Cat n°PYRU0002012)
Vitamines (Biomedia, Cat n° 1002012)
Beta Mercapto Ethanol (Promega, Cat n° H5081)

Buffer

PBS 1X (Biomedia, Cat n° PBS0002052)
Tris glucose (Tris buffer supplemented with 20% of glucose)

Cryoprotective agent

Dimethyl Sulfoxyde (DMSO) (Sigma, Cat n° D2650)

Agent of transfection:

Fugene 6 (Roche Cat n° 1 814 443)

Factors

4 different recombinant factors were used:

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❒ Recombinant Human Ciliary Neurotrophic Factor (CNTF) (R&D Cat n° 557-NT and Peprotech Inc, Cat n° 450-13)

❒ Recombinant IL11 (Peprotech, Cat n° 200-11)

❒ Recombinant Human Insulin Like Factor I (IGF1) (Peprotech Inc, Cat n° 100-11)

❒ Recombinant SCF (Peprotech, Cat n° 300-07)

Fetal Bovine Serum

Non Irradiated serum (PAA, Cat n° A15-022)

The batch used in this program was collected in Australia. This batch was added as supplement in the DMEM medium used for the culture of modified STO cells (feeder cells) and in Mac Coy'5A used for avian cell culture.

Dissociating agents

- ***Pronase*** (Roche, Cat n° 165 921)

Pronase is a recombinant protease manufactured by Roche Diagnostics, Germany, used for the dissociation of adherent avian stem cells.

1.2 - PROCESS OF ESTABLISHMENT OF CHICKEN EB1 CELL LINE

Eggs are opened, the yolk were separated from the albumen during the opening. The embryos were removed from the yolk directly with the aid of a Pasteur.

The chicken S86N embryos were placed in a tube containing physiological medium (1X PBS, Tris Glucose, medium, and the like). The embryos were then mechanically dissociated and inoculated on a layer of feeder STO cells into complete culture medium at 39°C (Figure 1). The feeder cells were seeded in dishes at around $2.7x10^4$ cell/cm^2. The complete culture medium is composed of basal commercial medium Mac Coy 5'A supplemented with 10% fetal calf serum, with IGF1, IL11, CNTF, SCF at a final concentration of 1ng/ml, and with 1% non-essential amino acids, with

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1% of mixture of vitamins of commercial origin, with sodium pyruvate at a final concentration of 1 mM, with beta-mercapto-ethanol at a final concentration of 0.2 mM, glutamine at a final concentration of 2.9 mM, with an initial mixture of antibiotics containing penicillin at a final concentration of 100 U/ml and streptomycin at a final concentration of 100 μg/ml.

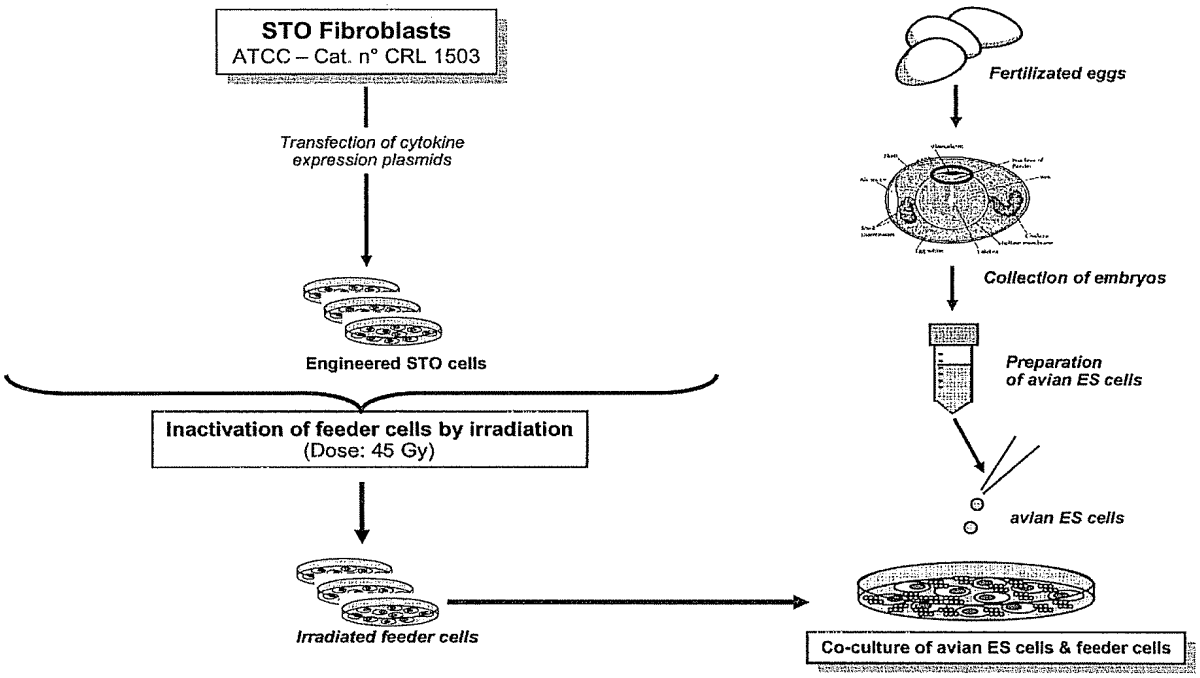

Figure 1: Principle of the embryo collection and co culture of ES cells on a feeder layer

When the avian ES cells from chicken embryos are passaged from a culture dish to another, the seeding of culture flasks was performed with around between 7 x $10^4/cm^2$ to 8 x $10^4/cm^2$ of avian ES cells in the complete culture medium. Preferably, the seeding is made with around 7.3 x $10^4/cm^2$ (4 x 10^6 cells/55cm^2 or 4 x 10^6 cells/100 mm dish). The avian cells, preferably the avian embryonic cells of step a) are cultured during several passages in the complete medium.

At passage 15, the complete medium was depleted in the 4 recombinant growth factors IGF1, CNTF, SCF and IL11. The depletion is made directly in one step, from one passage to another. The only factors maintained in

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the medium of culture, after passage 15, are produced by the modified STO cells (SGN and SCN cells) used as feeder layer.

After passage 32, the feeder layer producing factors in supernatant was replaced by non productive feeder cells. Deprivation of factors was then achieved after passage 32.

Cells were maintained between passage 32 to passage 111 in Mac Coy'5A supplemented with 10% FBS, vitamins, glutamine, Non essential amino acids, penicilline, streptomycine and Beta mercapto-ethanol.

At passage 111, the avian cells after dissociation were transfered directly in non cell culture treated dishes. The feeder deprivation was made in one step from one passage to another. Between passage 111 to passage 120, cell adherence was decreasing and more and more cells were observed in the supernatant. After passage 121, avian EB1 cells were seeded in non cell culture treated dishes and maintained under constant agitation in the incubator to avoid adhesion and improve the quality of the cell suspension.

After passage 135, serum deprivation was initiated, cells were transferred in a medium supplemented with 5% FBS, vitamins, glutamine, Non essential amino acids, penicilline, streptomycine and Beta-mercapto ethanol. After 10 passages of stabilization, cells were amplified and cryopreserved for further uses. EB1 cells which are small round diploid cells with a high nucleo-cytoplasmic ratio (Figure 5) display an embryonic-stem cell phenotype.

Figure 2 illustrates curve of proliferation of adherent and suspension EB1 cells along the process.

Figure 3A illustrates the cell phenotype of EB1 in adherence at passage P110 which corresponds approximately to 330 days into culture. Figure 3B illustrates the cell phenotype of EB1 in suspension at passage P133 which corresponds approximately to 400 days into culture.

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EB1 expresses alkaline phosphatase at late passage (passage 99 which corresponds approx. to 300 days into culture). Alcaline phosphatase is known to be specifically expressed in embryonic stem cells (Figure 4).

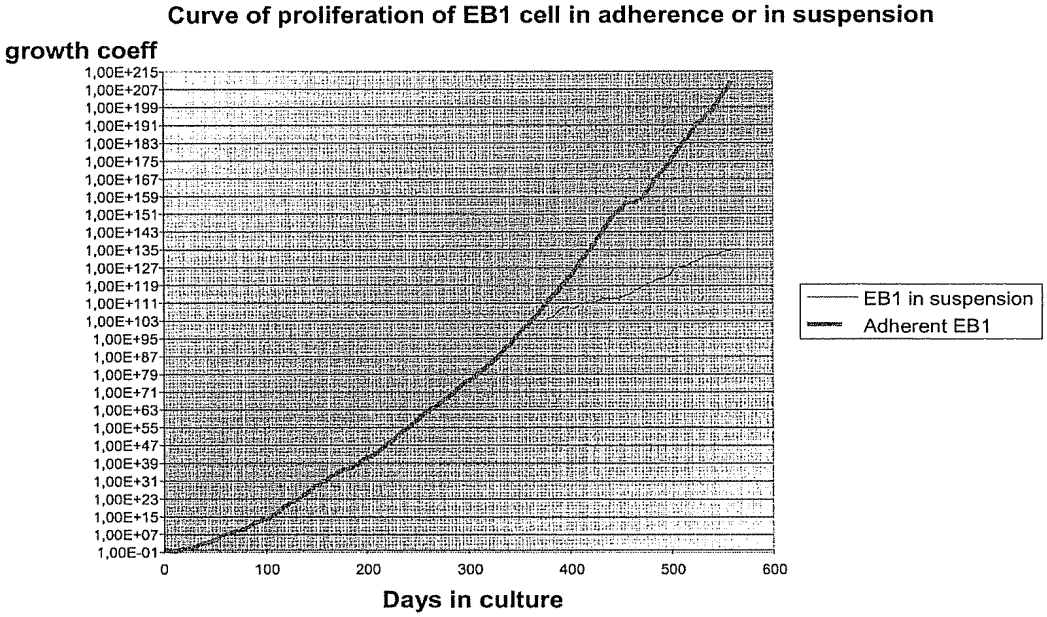

Figure 2: Curve of proliferation of adherent & suspension EB1 cells

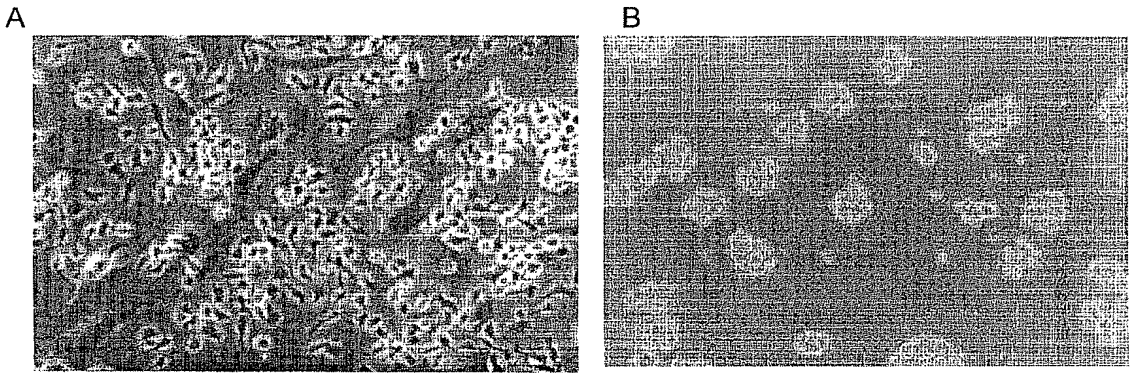

Figure 3 A & B: Phenotype of adherent EB1 cells at passage P110 (approx. 330 days in culture) (figure 3A) and EB1 in suspension at passage P133 (approx. 400 days in culture) (figure 3B)

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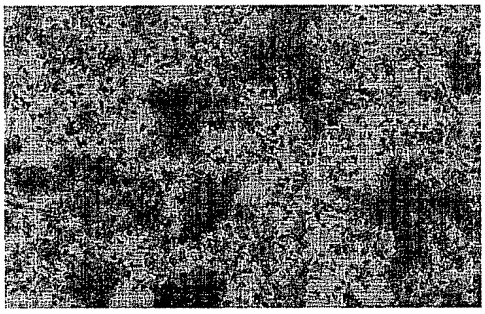

Figure 4: Alcaline phosphatase staining on adherent EB1 cells at passage 99 (approx. 300 days in cuture).

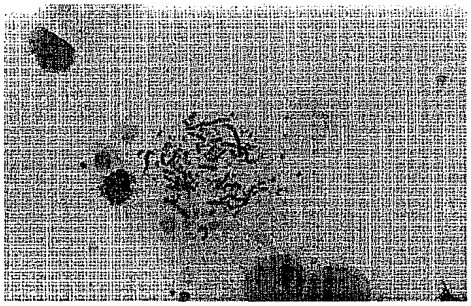

Figure 5: Karyotype analysis performed on adherent EB1 cells at passage 120: EB1 cells are diploid.

2. Etablishment of chicken cell EBs19 cell line

2.1 - RAW MATERIAL

Eggs

Eggs used for this project of development were produced by the S86N chicken strain produced by the Grimaud group. The S86N strain is the result of a cross between P6N females and S88N males. These eggs were not Specific Pathogen Free (SPF) eggs but, thanks a constant follow up of chicken flocks and strict procedures of access to production site, risk of cross contamination were under control. The chicken flocks producing eggs for this program of development were not vaccinated. Then, regularly sanitary testing were performed to check the SPF like status.

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Feeder cells

In the first step of the process of establishment of the EBs19 cell line, cells from murine origin (STO cells) were used as feeder layer to maintain the pluripotency of chicken stem cells. Those feeder cells are mitotically inactivated by gamma irradiation (45 to 55 Grays) before seeding on plastic. This dose of irradiation is a sub-lethal dose that induces a definitive arrest of the cell cycle but still permits the production of growth factors and extracellular matrix, necessary for the promotion of the cell growth of non differentiated cells.

The STO cell line was derived by A. Bernstein, Ontario Cancer Institute, Toronto, Canada from a continuous line of SIM (Sandos Inbred Mice) mouse embryonic fibroblasts and it was supplied by the American Type Culture Collection (ATCC) (STO Product number: CRL-1503, Batch number 3426890). Fresh feeder layers were prepared twice a week, in general on monday and thursday. Exponentially cells were dissociated and counted. A part of cells were seeded for maintenance of viable cultures and another part was irradiated. For irradiation, we prepared a cell suspension at $10x10^6$ cells/mL in tubes. Cells were exposed to a 45 to 55 grey dose and were seeded on plastic. After seeding, dishes or plates coated with inactivated feeder cells were used during a maximum of 5 days.

Medium

DMEM- HamF12 (Cambrex, Cat n° BE04-687)
DMEM (CAMBREX, Cat n° BE12-614F)
EX-CELL ^{TM} 65195 and 60947 (SAFC, customized medium)

Additives

Glutamine (Cambrex, Cat n° BE17-605E)
Pencillin/streptomycin (Cambrex, Cat n° BE17-602E))
Non essential Amino Acids (Cambrex, Cat n° BE13-114E)
Sodium pyruvate (Cambrex, Cat n°BE13-115)
Vitamines (Cambrex, Cat n° 13-607C)
Beta Mercapto Ethanol (Sigma, Cat n° M7522)

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Buffer and fixators

PBS 1X (Cambrex, Cat n° BE17-516F)

Cryoprotective agent

Dimethyl Sulfoxyde (DMSO) (Sigma, Cat n° D2650)

Factors

Two different recombinant factors were used:

❒ Recombinant Human Ciliary Neurotrophic Factor (CNTF) (Eurobio , Cat n°FC073803)

❒ Recombinant Human Insulin Like Factor I (IGF1) (Eurobio, Cat n° FC023803)

❒ Recombinant Stem Cell Factor (SCF) (Eurobio, Cat n° 053803)

❒ Recombiannat basic Fibroblast Growth Factor (bFGF) (Eurobio, Cat n° FC013803)

❒ Recombiant IL6 (Eurobio, FC033803)

❒ Recombinant IL6r (Eurobio, FC063803)

Fetal Bovine Serum

Non irradiated Fetal Bovine Serum (FBS) (JRH, Cat n° 12103)

The non irradiated serum used in the program was collected and produced in United States. Animals used for collection were USDA inspected and acceptable for slaughter. It was added in the medium during avian stem cells culture. This batch was not submitted to irradiation to avoid the destruction of critical proteins or components identified as essential for the maintenance of stem cells in culture.

Irradiated serum (JRH, Cat n° 12107)

The irradiated batch used in this program was also collected in United States. This irradiated batch was added as supplement in the DMEM medium used for the culture of STO or FED cells (feeder cells). Those cells do not require as stem cells a specific quality of serum for growth and maintenance in culture. To minimize high concentration of serum in the

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medium we have adapted the STO cells to grow in presence of 4% of FBS only.

Dissociating agents

- ***Pronase*** (Roche, Cat n° 165 921)

Pronase is a recombinant protease manufactured by Roche Diagnostics, Germany, used for the dissociation of adherent avian stem cells.

2.2 - PROCESS OF ESTABLISHMENT OF CHICKEN EBs19 CELL LINE

Eggs are opened, the yolk were separated from the albumen during the opening. The embryos were removed from the yolk with the aid of a small absorbent filter paper (Whatmann 3M paper), cut out beforehand in the form of a perforated ring with the aid of a punch. The diameter of the perforation were about 5 mm. These small rings were sterilized using dry heat for about 30 minutes in an oven. This small paper ring is deposited on the surface of the yolk and centered on the embryo which is thus surrounded by the paper ring. The latter is then cut out with the aid of small pairs of scissors and the whole removed is placed in a Petri dish, filled with PBS or with a physiological saline. The embryo thus carried away by the ring were cleaned of the excess yolk in the medium and the embryonic disk, thus free of the excess vitellin, is collected with a Pasteur pipette.

The chicken S86N embryos were placed in a tube containing physiological medium (1X PBS). The S86N embryos were then mechanically dissociated and inoculated on a layer of feeder STO cells into complete culture medium at 39°C. The feeder cells were seeded in flask at around $2.7x10^4$ cell/cm^2. The complete culture medium is composed of basal commercial medium DMEM-Ham F12 supplemented with 10% fetal calf serum, with IGF1, CNTF, bFGF, SCF, IL6, IL6r at a final concentration of 1ng/ml, with 1% non-essential amino acids, with 1% of mixture of vitamins of commercial origin, with sodium pyruvate at a final concentration of 1 mM, with beta-mercapto-ethanol at a final concentration of 0.2 mM, glutamine at a final concentration of 2.9 mM, with an initial mixture of antibiotics containing penicillin at a final concentration of 100 U/ml and streptomycin

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at a final concentration of 100 μg/ml. Rapidly after the first passages of the cells, the mixture of antibiotics is no longer added to the medium.

When the avian ES cells from chicken Valo embryos is passaged from a culture flask to another, the seeding of culture flasks was performed with around between 7 x 10^4/cm^2 to 8 x 10^4/cm^2 of avian ES cells in the complete culture medium. Preferably, the seeding is made with around 7.3 x 10^4/cm^2 (4 x 10^6 cells/55cm^2 or 4 x 10^6 cells/100 mm dish). The avian cells, preferably the avian embryonic cells of step a) are cultured during several passages in the complete medium. At passage 5, the complete medium was depleted in antibiotics and 4 growth factors: SCF, bFGF, IL6 & IL6r. The depletion is made directly in one step, from one passage to another. Until passage 10, the avian embryonic cells were cultured in the medium supplemented only with IGF1 and CNTF growth factors. At passage 10, IGF1 and CNTF were depleted and cells were maintained in the medium free of recombinant factors.

Then depletion of feeder cells were performed after the depletion of growth factors IGF1 and CNTF by a progressive decreasing of feeder cells concentration over several passages. The feeder deprivation was initiated at passage 27 and achieved at passage 69. Practically, the same concentration of the feeder cells were used for 3 to 7 passages, then a lower concentration of the feeder cells were used for an additional 3 to 7 passages, and so on. The flask were originally seeded with around 2.7 x10^4 feeder cells/cm^2, then after passage 27 with around 2.2 x 10^4 feeder cells/cm^2, then after passage 33 around 1.8 x 10^4 feeder cells/cm^2, then after passage 39 with around 1.4 x 10^4 feeder cells/cm^2, then after passage 43 with around 1.1 x 10^4 feeder cells/cm^2, then after passage 51 with around 1 x 10^4 feeder cells/cm^2, then after passage 59 with around 0.9 x 10^4 feeder cells/cm^2, then after passage 66 with around 0.5 x 10^4 feeder cells/cm^2 and finally no feeder cells were used after passage 69. Between passage 69 and passage 120, cells were maintained without feeder layer for cell line stabilization (Figures 6 & 7). Figure 6 illustrates the cell proliferation of EBs19 cell line along the factor and the feeder deprivation.

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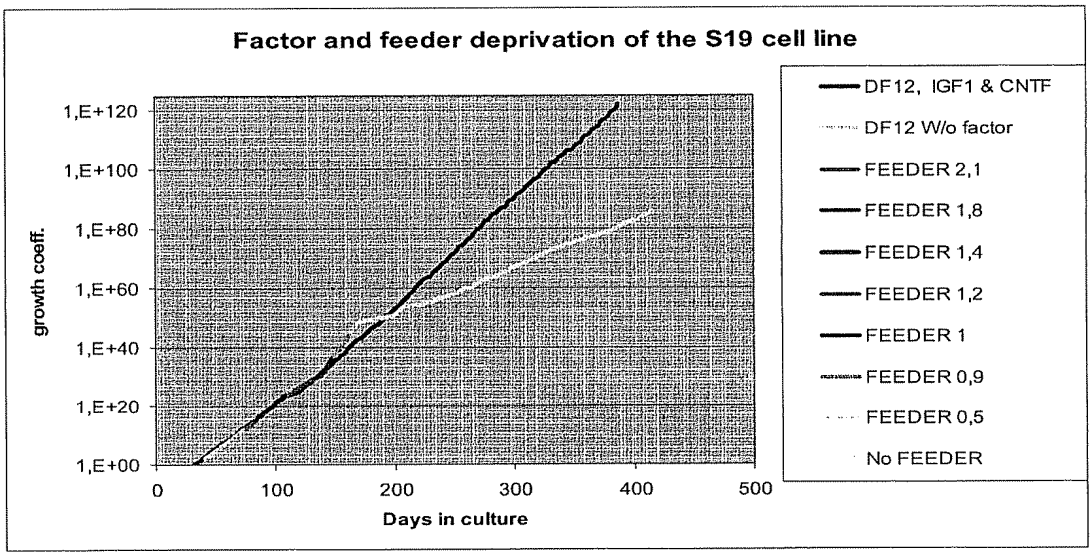

Figure 6: Curve of proliferation of the EBs19 cell line along factor and feeder deprivation

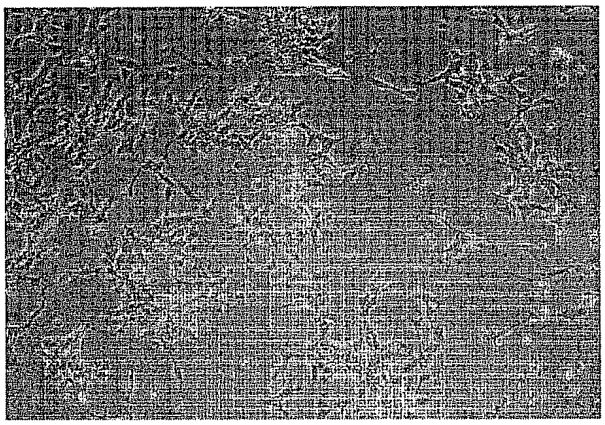

Figure 7: EBs19 cells growing with animal serum and without feeder cells at passage 71

The serum depletion was performed after the growth factor and the feeder cells depletion. At the beginning of serum depletion, the culture medium were composed of basal commercial medium DMEM-HamF12 supplemented with 10% fetal calf serum and with 1% non-essential amino acids, with 1% of mixture of vitamins of commercial origin, with sodium pyruvate at a final concentration of 1 mM, with beta-mercaptoethanol at a final concentration of 0.2 mM, glutamine at a final concentration of 2.9 mM.

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The chicken S19 cells were adapted to the growth in a serum free medium culture in a two steps process: first, the chicken S19 cells were rapidly adapted to a culture medium composed of commercial serum free medium (SFM), ExCell 60947 (SAFC Biosciences) supplemented with 5% fetal calf serum. Once this rapid adaptation to the new medium was performed, a second step was initiated consisting of a slow adaptation to decreasing concentration of animal serum in the SFM medium. Starting from 5% serum, the cells were transferred in medium supplemented with 2.5%, then 1.25%, and finally 0%. Serum depletion started at passage 120 and ended at passage 155.

Cells were maintained at 39°C in SFM until passage 222. After passage 222, cell adaptation to a lower temperature of culture (37°C) was performed on 4 passages. Finally after passage 226, EBs19 cells were able to proliferate at 37°C without growth factor, without feeder cells and without serum. Cells were maintained in those culture conditions until passage 233. At passage 233, cells were cryo-preserved with a PDT (Population Doubling Time) of approximately 24 Hours. Figure 8 illustrates the growth curve of EBs19 cell lines along the serum deprivation and the low temperature adaptation.

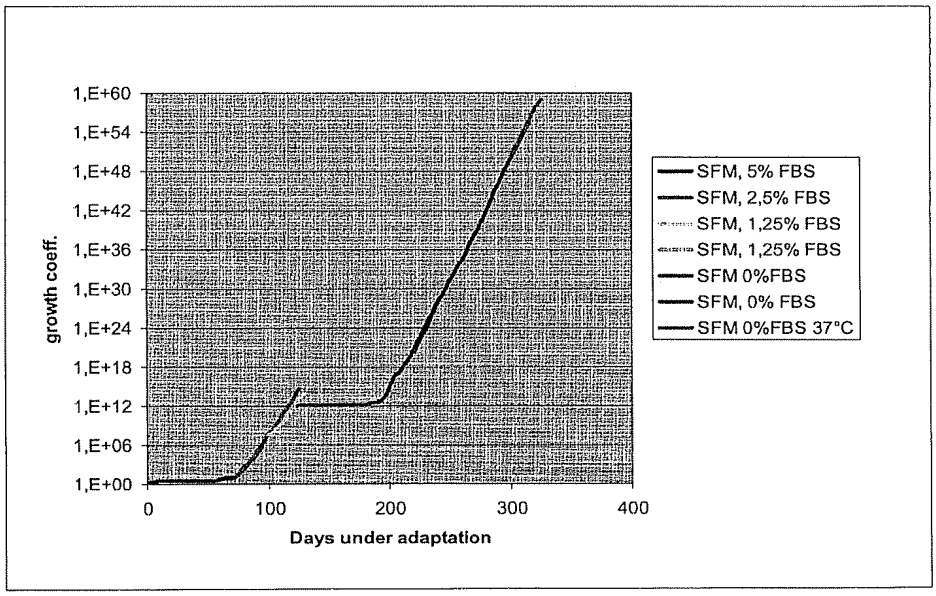

Figure 8: Serum deprivation and low temperature adaptation of the EBs19 cell line

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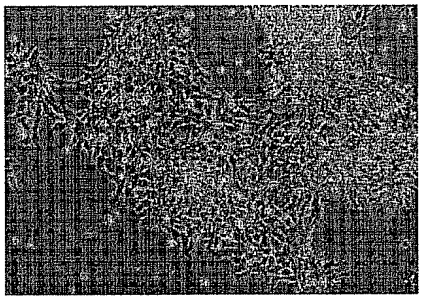

Figure 9: EBs19 cells growing without factor, without feeder cells and without serum at passage 233 (corresponding approximately to 600 days of culture)

9. In my opinion, these experiments show the cell lines produced by the claimed method possess the ability to proliferate over a long period of time in the absence of exogenous growth factors.

10. I further declare that all statements made herein of my own knowledge are true and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issuing thereon.

Date: April 28, 2008 By: GUEHENNEUX Fabienne